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Influence of Dissolved Organic Matter Sources on In-Stream Net Dissolved Organic Carbon Uptake in a Mediterranean Stream

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Abstract: Studies exploring how different sources of dissolved organic matter (DOM) influence in-stream dissolved organic carbon (DOC) uptake at the ecosystem scale are scarce in the literature. To fill this knowledge gap, we examined the relationship between DOM sources and in-stream net DOC uptake (U_{DOC}) in a sub-humid Mediterranean stream. We considered four reach-scale scenarios occurring under natural conditions that differed in predominant DOM sources (groundwater, leaf litter, and/or upstream water). Results showed that groundwater inputs favored in-stream net DOC uptake, while leaf litter inputs promoted in-stream net DOC release. However, there was no clear effect of DOM source mixing on the magnitude of U_{DOC}. Further, the variability in U_{DOC} within and among scenarios was mostly explained by stream DOC concentration, suggesting that DOC availability limits microbial activity in this stream. DOM composition became a controlling factor of U_{DOC} variability only during the leaf litter period, when stream DOC concentration was the highest. Together, these results suggest that the capacity of headwater forested streams to process DOC is closely tied to the availability of different DOM sources and how they vary over time and along the river network.

Keywords: dissolved organic carbon; in-stream net uptake; leaf litter; groundwater inputs; dissolved organic matter composition; carbon availability

1. Introduction

Streams and rivers retain and transform large amounts of particulate and dissolved organic carbon (DOC) on its downstream route to marine ecosystems [1–3]. In-stream uptake of terrestrial DOC exports is central to many biogeochemical and ecological process in streams [4,5] and, as such, several studies have focused on quantifying its rates across ecosystems [1,6,7]. Yet, the drivers of in-stream DOC uptake over time and across streams still remain unclear, which limits our ability to integrate the role of freshwaters on the global biogeochemical cycles.

The source of dissolved organic matter (DOM) strongly influences in-stream DOC uptake by determining the composition of DOM materials [7,8]. In-stream primary producers (e.g., algae) generally release an array of highly reactive biopolymers [9], whereas terrestrially-derived DOM typically contains a higher proportion of aromatic and humic compounds [10]. The humic character of terrestrial DOM is mostly associated with leachates from riparian leaf litter [11] or with groundwater draining wetlands and boreal forests [12], but this does not seem to be universal. For example, groundwater inputs draining poorly-developed soils mostly supply protein-like DOM to streams [13,14]. In addition to the individual nature of different DOM sources, laboratory experiments have shown that the diversity of DOM sources can also favor in-stream DOC uptake, likely by increasing the array of DOM compounds available for microbes [15–17]. However, field studies show no clear trends on the effects of DOM sources diversity on in-stream DOM uptake [18]. These contradictory results may be partially explained by the complexity of measuring the effect of a mixture of DOM sources on DOC uptake in the field, because both the composition of a given DOM source and its relative contribution to the stream DOM pool vary over time and along fluvial networks [5].

Mediterranean streams show high spatial and temporal variability in both stream DOC concentration and DOM composition that can be associated with the relative dominance of different DOM sources. Riparian leaf litter inputs during fall commonly lead to increases in stream DOC concentration and dominance of humic-like DOM compounds [14,19]. Increases in stream DOC concentration are also typical of periods with high hydrological connectivity as a consequence of large terrestrially-derived DOM inputs via groundwater flowpaths [20,21]. Conversely, low in-stream DOC concentration and a high protein-like fraction of DOM typically occur during periods of low hydrological connectivity. This phenomenon is attributed to an increase in the relevance of in-stream DOM production, especially in those streams or reaches that lose water towards adjacent riparian zones [20,22]. Hence, the spatial and temporal variability of both leaf litter inputs and terrestrial–aquatic hydrological linkages observed in Mediterranean streams can generate a dynamic set of scenarios that can be leveraged to test the influence of DOM sources (alone or in combination) on in-stream DOC uptake.

The objective of this study was to examine the variability of in-stream net DOC uptake (U_{DOC}) in a sub-humid Mediterranean forested stream and to explore if this variability is related to contrasting contributions of DOM sources to the stream. To do so, we estimated U_{DOC} in 14 contiguous reaches along a 3.7 km section of a headwater, forested stream on 10 dates over 1.5 years. This study design allowed for sampling stream water under different scenarios for which the contribution of leaf litter and riparian groundwater inputs to stream DOM strongly differed. Specifically, considering the relative importance of the different DOM sources, we identified four scenarios (Figure 1): (1) Reaches mostly receiving DOM from upstream water, (2) reaches receiving DOM from groundwater and upstream water, (3) reaches receiving DOM from leaf litter and upstream water, and (4) reaches receiving DOM from leaf litter, groundwater, and upstream water. We expected that U_{DOC} will vary among DOM scenarios and will increase with increasing diversity of DOM sources. Hence, U_{DOC} will be higher when all sources (i.e., leaf litter, groundwater, and upstream water) contribute to stream DOM. Finally, we investigated the factors driving U_{DOC} variability by exploring the relationship between U_{DOC}, stream DOC concentration, and DOM composition, and other essential environmental variables, such as groundwater discharge, nutrient availability, and stream temperature.

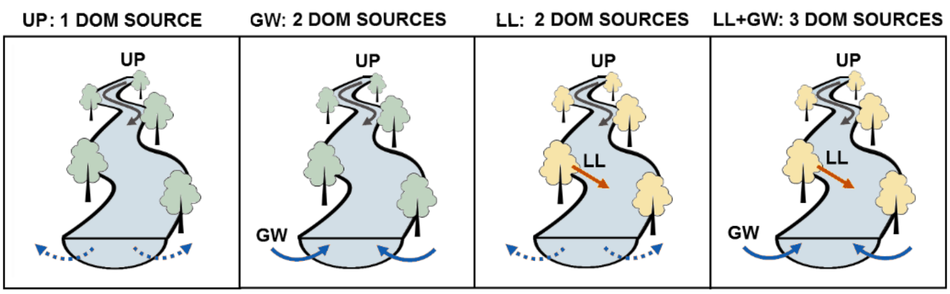

Figure 1. Conceptual figure of the four scenarios. The scenario UP includes those reaches receiving dissolved organic matter (DOM) mostly from upstream water (indicated with the grey arrow "UP"). The scenario GW includes those reaches receiving DOM from groundwater (indicated with the blue arrow "GW") and upstream water. The scenario LL includes those reaches receiving DOM from leaf litter (indicated with the orange arrow "LL") and upstream water. Finally, the scenario LL + GW includes those reaches receiving DOM from leaf litter, groundwater, and upstream water.

2. Methods

2.1. Study Site

The study was conducted in the Font del Regàs catchment (14.2 km^2), located in the Montseny Natural Park, NE Spain (41°50′ N, 2°30′ E, 300–1200 m above the see level). The catchment is dominated by biotitic granite and has steep slopes (28%). Evergreen oak (*Quercus ilex*) and beech (*Fagus sylvatica*) forests cover 54% and 38% of the catchment area, respectively (Figure 2). Population density within the catchment is low (<1 person/km^2). The riparian zone is relatively flat (slope < 10%), and it covers 6% of the catchment area. *Alnus glutinosa*, *Robinia pseudoacacia*, *Platanus hybrida*, and *Fraxinus excelsior* are the most abundant riparian tree species. The climate is sub-humid Mediterranean, with mild winters and dry summers. During the study period, annual precipitation and temperature averaged 975 mm and 12.9 °C, respectively.

The Font del Regàs stream is a perennial third-order stream. At the headwaters, the streambed is mainly composed of rocks and cobbles (70%), with a small contribution of sand (~10%). At the valley bottom, sands and gravels represent 44% of the streambed substrate and the presence of rocks is minor (14%). On average, stream discharge increases from headwaters (23 ± 17 L s^{-1}) to the valley bottom (105 ± 113 L s^{-1}; period 2010–2013), indicating that the stream tends to gain water along the catchment. Yet, there are discrete net hydrologically loosing reaches along the mainstem, especially during summer [23]. Permanent tributaries comprise about 50% of the catchment area and contribute 56% of stream discharge [23].

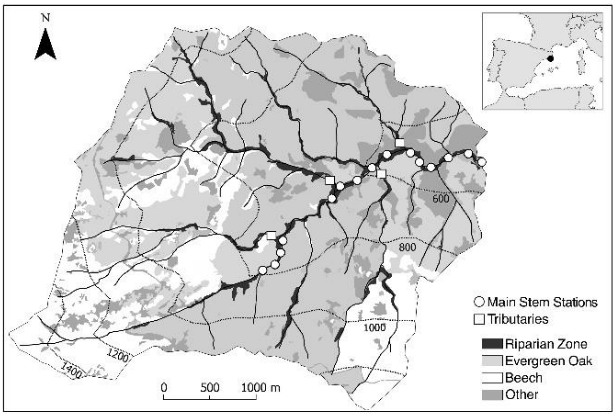

Figure 2. Map of the Font del Regàs catchment (NE Spain). The map shows the vegetation cover and the stream sampling sites along the 3.7 km section (n = 15). Four permanent tributaries discharge into the stream and were also sampled (white squares). The remaining tributaries were consistently dry during the study period. The inset shows the location of the Font del Regàs catchment within Spain.

2.2. Field Sampling

We selected 15 sampling sites along a 3.7 km section of the Font del Regàs stream. Sampling sites were located from 110 to 600 m apart from each other and resulted in 14 contiguous reaches (Figure 2). At these sites, we collected stream water (from the thalweg) and riparian groundwater (from 1 m long piezometers located at ~1.5 m from the stream channel edge) every two months from October 2010 to December 2011 (10 sampling dates). Groundwater samples were collected with a 100 mL syringe connected to a silicone tube. We assumed this water to be representative of the groundwater entering the stream. Water samples were collected with pre-acid-washed polyethylene bottles after triple-rinsing them with either stream water or groundwater. All field campaigns were conducted under base flow conditions, when the influence of in-stream processes on C cycling was assumed to be relevant. At each sampling site, we also measured water conductivity and temperature with a WTW-3310 (Welheim, Germany), and stream discharge (Q, in L s^{-1}). Q was measured by adding 1 L of sodium chloride-enriched solution to the stream and using a mass balance approach [24].

For comparison purposes among stream reaches, Q was normalized by the corresponding drainage area at each sampling site ($\hat{Q}$ in L s^{-1} km^{-2}). On each sampling date, we also collected stream water and measured Q at the four permanent tributaries discharging into Font del Regàs stream (Figure 2). These data were used to estimate reach-scale DOC mass balances along the mainstem of the study stream section (see below).

2.3. Laboratory Analysis

Water samples were filtered through pre-ashed GF/F filters (Whatman®, Maidstone, UK) and kept cold (<4 °C) until laboratory analysis (<24 h after collection). DOC and total dissolved nitrogen (TDN) concentrations were determined using a Shimadzu TOC-VCS coupled to a total nitrogen analyzer (Kyoto, Japan). Concentration of DOC was determined by oxidative combustion infrared analysis and concentration of TDN by oxidative combustion-chemiluminescence. Concentrations of ammonium (NH_4), nitrate (NO_3), and soluble reactive phosphorous (SRP) were determined by standard colorimetric methods (details in Reference [23]). Note that NO_3 concentrations accounted for both NO_3 and nitrite, yet the concentrations of the latter were generally below the detection limit. Concentration of dissolved organic nitrogen (DON) was calculated by subtracting NO_3 and NH_4 concentrations from TDN concentration.

We used different metrics to assess stream DOM composition. Fluorescence excitation-emission spectra were recorded on a Shimadzu RF-5301 PC spectrofluorimeter (Kyoto, Japan) over an emission range of 270–700 nm (1 nm steps) and an excitation range of 230–430 nm (10 nm steps) (Supplementary Figure S1). Details on the measurements and corrections made can be found in Bernal et al. [14]. We calculated three spectroscopic metrics: the fluorescence index (FI) [25], the biological index (BIX) [26], and the humification index (HIX) [27]. The FI index is linked to the DOM origin, with low and high values being characteristic of terrestrial plant and microbial DOM sources, respectively [25]. The BIX index is linked to the aging of DOM, with high values indicating a higher contribution of recently produced DOM; for instance, from microbial activity in the stream [26]. The HIX index is a proxy of the humification degree of DOM, with higher values indicating higher humification degree [28].

We used a Parallel Factor Analysis (PARAFAC) to identify the main fluorescence components of DOM [29]. The analysis was performed using the DrEEM toolbox for MATLAB (Mathworks, Inc., Natick, MA) [30]. The PARAFAC modelling of Excitation-Emission Matrix (EEM) spectra revealed four independent components in the analyzed samples (see Reference [14]). Components C2 and C3 corresponded to humic-like materials, while components C1 and C4 corresponded to protein-like fluorescence. Given the nature of the components, we grouped them into humic-like (H_{DOM}: sum of C2 and C3) and protein-like (P_{DOM}: sum of C1 and C4). In addition, we extracted the values of the most commonly observed fluorescence peaks in freshwater DOM [28]. Details on the analysis, identification of PARAFAC components, and validation process can be found in Bernal et al. [14].

2.4. Calculation of In-Stream Net DOC Uptake

We estimated U_{DOC} (in µg m^{-1} s^{-1}) by applying a mass balance approach for each stream reach. The mass balance included all hydrological input (i.e., upstream, tributaries, and groundwater) and output (i.e., downstream export) fluxes. For each sampling date, U_{DOC} was approximated as follows:

$$U_{DOC} = (Q_{UP} \times C_{UP} + Q_{GW} \times C_{GW} + Q_{TR} \times C_{TR} - Q_{DW} \times C_{DW})/L \quad (1)$$

where Q_{UP} and Q_{DW} are the discharge at the upstream and downstream ends of each reach, respectively. Q_{TR} is the discharge from tributaries (when present), and Q_{GW} is the discharge from groundwater in net terms (all in L s^{-1}). Q_{GW} was estimated as the difference between Q_{DW} and Q_{UP} and has either positive or negative values, which are indicative of hydrologically net gaining or losing reaches, respectively. C_{UP} and C_{DW} are the stream water DOC concentration measured at the upstream and downstream ends of the reach respectively, while C_{TR} and C_{GW} are the DOC concentration measured at tributaries and riparian groundwater, respectively (all in μg C L^{-1}). For net gaining reaches ($Q_{GW} > 0$), C_{GW} averaged groundwater concentration measured at the upstream and downstream ends of the reach. For net losing reaches ($Q_{GW} < 0$), C_{GW} averaged stream water concentration measured at the upstream and downstream ends of the reach. Finally, L is the length of the reach (in m). We calculated an upper and lower limit of U_{DOC} based on the empirical uncertainty associated with Q and DOC measurements, which was relatively small (2–3%) [23].

We considered that $U_{DOC} > 0$ indicates that gross DOC uptake prevails over release, $U_{DOC} < 0$ indicates that DOC release prevails over gross uptake, and $U_{DOC} \sim 0$ indicates that gross DOC uptake ~ DOC release [14]. Therefore, we expected $U_{DOC} \neq 0$ if DOC does not behave conservatively and in-stream processes contributing to gross DOC uptake and release do not fully counterbalance each other. We assumed that U_{DOC} was indistinguishable from 0 when the range of upper and lower limits contained zero.

2.5. Data Analysis

In order to explore the influence of different DOM sources (i.e., upstream, leaf litter, and groundwater) on U_{DOC}, the dataset (14 reaches x 10 dates) was divided into the leaf litter fall (October–December) and the no leaf litter fall (January–September) periods. In addition, for each period, we divided the dataset based on the predominant direction of water at the riparian–stream interface in each reach (net gaining versus net losing). The cases for which Q_{GW} was indistinguishable from zero (28 out of 140) were excluded from posterior analysis. As a result of this classification, we obtained four scenarios with distinct DOM sources (Figure 1). The scenario "UP" included those reaches that received DOM inputs mostly from upstream water, that is all losing reaches during the no leaf litter period (n = 31). The scenario "GW" included those reaches that received DOM inputs from both groundwater and upstream water and comprised all gaining reaches during the no leaf litter period (n = 36). The scenario "LL" included those reaches that had both leaf litter and upstream water as major DOM sources, that is all losing reaches during the leaf litter period (n = 18). Finally, the scenario "LL + GW" included those reaches that received DOM inputs from leaf litter, groundwater, and upstream water. This last scenario comprised all gaining reaches during the leaf litter period (n = 27).

We applied a Mann–Whitney test to explore whether stream water temperature, hydrological variables (Q, $\hat{Q}$, Q_{GW}), stream water concentrations (NO_3^-, NH_4^+, SRP, DON, and DOC), and DOM spectroscopic metrics (FI, BIX, HIX, H_{DOM}, and P_{DOM}) differed among the four scenarios. We used non-parametric tests because datasets were relatively small, not normally distributed, and showed heteroscedasticity [31]. In all cases, differences were considered significant if $p < 0.05$. Further, to test the variability of stream DOM composition among and within scenarios, we performed a principal component analysis (PCA) on the spectroscopic metrics and PARAFAC components (function *princomp* of base R). We used the scores of each sample for the two first principal components (PC) to calculate the centroid and the standard deviation of each scenario for each PC axis. For each axis, the distance between centroids indicate relative differences in DOM composition among scenarios, while the standard deviation indicates the degree of variability in DOM composition associated with each axis for each scenario. Prior to the PCA analysis, data was scaled and centered.

We also applied a Mann–Whitney test to explore whether U_{DOC} differed among the four scenarios. For each scenario, we further calculated the proportion of U_{DOC} values that were >0, <0, and =0, and tested whether these proportions were statistically different among scenarios by using a contingency-table analysis [31]. When differences were statistically significant, we applied a Tukey test to determine which scenarios were different from each other [31].

For each scenario and for all data pooled together, we also performed linear mixed regression models to examine the contribution of stream DOC concentration and DOM composition on the variability of U_{DOC}. As a first step, we considered these variables as fixed effects, while we set time and distance (the latter as a proxy of location of each reach along the study section) as random effects

(R package *lme4*). We selected the best-fit model by applying a step-wise analysis (*step* function, *lmerTest* package in R). For each model, we tested the independence of variables using autocorrelation models (*acf* function, *rioja* package in R). We also tested residuals for normality using a Shapiro–Wilk test and examined the homogeneity of variance by plotting the predicted and residual values. Finally, we computed the partition of variance of each selected variable from the resulting incremental sums of squares table [31]. In order to test the influence of additional environmental factors on U_{DOC} variability, we repeated the multiple linear mixed regressions including all the hydrological, physical, and chemical variables described in Table 1 as fixed effects. All statistical tests were run with R 3.5.3 [32].

3. Results

3.1. Characterization of DOM Source Scenarios

The four scenarios showed no differences in Q, which averaged 72 L s^{-1}. However, significant differences were found in $\hat{Q}$ for which the highest value was measured in the LL + GW scenario (average = 10 L s^{-1} km^{-2}) (Table 1). Regarding groundwater discharge, the two scenarios defined by gaining reaches (GW and LL + GW) showed similar mean values for Q_{GW} (15 ± 4 L s^{-1}). Likewise, the two scenarios defined by losing reaches (UP and LL) showed negative Q_{GW} values of similar magnitude (average Q_{GW} = −14 ± 3 L s^{-1}). There were also differences in the physicochemical signature of the four scenarios. Stream water temperature was lower during LL and LL + GW scenarios than during the UP scenario (Table 1), while stream DOC concentration was higher and more variable during the leaf litter period (scenarios LL and LL + GW; Table 1, Supplementary Figure S2). Stream nutrient concentrations were not statistically significant among the four scenarios.

Table 1. Hydrological and chemical characterization of the four scenarios. Values are means ± standard deviations for each scenario. For each variable, different letters indicate differences among scenarios (Mann–Whitney test). Scenarios are: mostly upstream DOM inputs (UP, n = 31), groundwater and upstream DOM inputs (GW, n = 36), leaf litter and upstream DOM inputs (LL, n = 18), and leaf litter, groundwater, and upstream DOM inputs (LL + GW, n = 27). Abbreviations: stream discharge (Q); stream discharge normalized by catchment area ($\hat{Q}$); groundwater discharge (Q_{GW}); stream water temperature (Temp); stream concentrations of nitrate (NO_3), ammonium (NH_4), soluble reactive phosphorous (SRP), dissolved organic nitrogen (DON), and dissolved organic carbon (DOC); fluorescence index (FI); biological index (BIX); humification index (HIX); sums of protein-like (P_{DOM}) and the humic-like (H_{DOM}) components resulting from the Parallel Factor Analysis (PARAFAC). Note that P_{DOM} and H_{DOM} are also expressed as the relative contribution of protein- or humic-like components respectively, to the total sum of components.

Variable	UP	GW	LL	LL + GW
Discharge and temperature				
Q (L s^{-1})	54 ± 39 ^{A}	62 ± 46 ^{A}	85 ± 59 ^{A}	78 ± 53 ^{A}
$\hat{Q}$ (L s^{-1} km^{-2})	6 ± 4 ^{A}	8 ± 5 ^{AB}	7 ± 5 ^{AB}	10 ± 6 ^{B}
Q_{GW} (L s^{-1})	−12 ± 15 ^{A}	14 ± 20 ^{B}	−17 ± 15 ^{A}	15 ± 24 ^{B}
Temp (°C)	12.3 ± 3.7 ^{A}	11.4 ± 4.0 ^{AB}	8.9± 2.1 ^{B}	8.8 ± 1.8 ^{B}
Stream water chemistry				
NO_3 (µg N L^{-1})	187 ± 71 ^{A}	202 ± 56 ^{A}	236 ± 145 ^{A}	272 ± 149 ^{A}
NH_4 (µg N L^{-1})	11 ± 5 ^{A}	11 ± 3 ^{A}	8 ± 4 ^{A}	9 ± 4 ^{A}
SRP (µg P L^{-1})	14 ± 6 ^{A}	13 ± 9 ^{A}	12 ± 6 ^{A}	13 ± 7 ^{A}
DON (µg N L^{-1})	65 ± 78 ^{A}	52 ± 32 ^{A}	138 ± 357 ^{A}	49 ± 39 ^{A}
DOC (µg C L^{-1})	403 ± 139 ^{A}	435 ± 196 ^{A}	1644 ± 1703 ^{B}	1161 ± 1070 ^{B}
Spectroscopic metrics				
FI	2.60 ± 0.10 ^{A}	2.50 ± 0.10 ^{A}	2.80 ± 0.40 ^{B}	2.80 ± 0.60 ^{B}
BIX	0.67 ± 0.08 ^{A}	0.70 ± 0.20 ^{A}	0.62 ± 0.11 ^{A}	0.63 ± 0.11 ^{A}
HIX	1.01 ± 0.23 ^{A}	0.94 ± 0.25 ^{A}	1.08± 0.55 ^{A}	0.98 ± 0.43 ^{A}
H_{DOM}	0.60 ± 0.11 ^{A}	0.55 ± 0.10 ^{A}	0.94 ± 0.39 ^{B}	0.85 ± 0.30 ^{B}
	27% ± 6%	26% ± 6%	26% ± 7%	35% ± 12%
P_{DOM}	1.92 ± 0.60 ^{A}	1.85 ± 0.49 ^{A}	2.79 ± 1.25 ^{B}	2.47 ± 1.27 ^{AB}
	73% ± 14%	74% ± 13%	74% ± 7%	65% ± 25%

In all scenarios, stream DOM was characterized by a strong protein-like signature, with relatively high FI values (>2), low HIX (~1), and a dominance of the P_{DOM} group of components, which represented >60% of total DOM fluorescence (Table 1). However, there were remarkable differences in the DOM composition among the four scenarios, which were mostly associated with leaf litter inputs. In particular, FI and H_{DOM} were statistically higher in scenarios covering the leaf litter fall period (scenarios LL and LL + GW) than in those covering the period with no leaf litter inputs (scenarios UP and GW) (Table 1, Supplementary Figure S2). Further, P_{DOM} was statistically higher in the LL scenario than during the UP and GW scenarios.

Results from the PCA further indicated differences in DOM composition among the four scenarios. The first PCA component (PC1) explained 49.9% of the total variance and was related to the humic- versus protein-like character of DOM. Positive loadings in PC1 were related to humic-like components C2 and C3 and humic peaks A and C, while negative loadings were related to the protein-like peak B and components C1 and C4 (Figure 3A). The second PCA component (PC2) explained 19.4% of the total variance and was related to different aspects of the protein-like character of DOM. Positive loadings in PC2 were related to the tyrosine-like component C1, while negative loadings were related to the tryptophan-like Peak T and BIX (Figure 3A). The centroids of the four scenarios were located close to zero, though they fall in different quadrants. The centroids of the two scenarios with leaf litter inputs (LL and LL + GW) fall in the positive side of PC1 and PC2, indicating a higher contribution of humic-like materials in those samples and a predominance of tyrosine-like protein compounds. Moreover, samples within the LL and LL + GW scenarios showed a higher variability (i.e., higher standard deviation) along PC1, denoting a higher diversity of humic compounds during the leaf litter period (Figure 3B). In contrast, the centroid of the two scenarios without leaf litter inputs (UP and GW) fall in the negative side of PC1 and PC2, implying a predominance of protein, tryptophan-like compounds in those samples. Moreover, samples within the UP and GW scenarios showed high variability along the PC2 axis, indicating higher diversity of protein-like materials during the period without leaf litter inputs (Figure 3B).

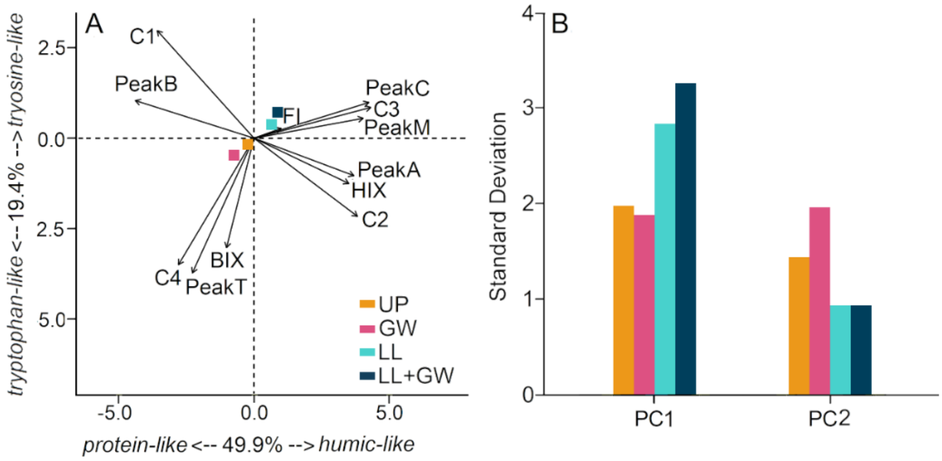

Figure 3. Principal component analysis (PCA) of dissolved organic matter (DOM) spectroscopic metrics. (**A**) Representation of the PC1 (x-axis) and PC2 (y-axis). The length of the arrows indicates the relative importance of each variable to each PC. Symbols indicate the centroid of each scenario. The distance among centroids indicates average differences in DOM composition among scenarios. (**B**) Standard deviation of the scores for cases from each scenario for PC1 and PC2. The higher the standard deviation, the larger the variability in DOM composition within a particular PC for a given scenario. Scenarios are: mostly upstream DOM inputs (UP), groundwater and upstream DOM inputs (GW), leaf litter and upstream DOM inputs (LL), and leaf litter, groundwater and upstream DOM inputs (LL + GW). Abbreviations: fluorescence index (FI); biological index (BIX); humification index (HIX); components resulting from the PARAFAC analysis (C1–C4); humic-like peaks (Peaks A and C); tyrosine-like peak (Peak B); processed humic-like peak (Peak M); tryptophan-like peak (Peak T).

3.2. Comparison of In-Stream Net DOC Uptake among DOM Source Scenarios

During the study period, mean U_{DOC} considering all datasets together equaled 16.5 ± 13.5 μg C m^{-1} s^{-1}. Mean U_{DOC} was negative for the UP (−1.4 ± 29.4 μg C m^{-1} s^{-1}) and LL scenarios (−26.5 ± 90.1 μg C s^{-1} m^{-1}), but positive for the GW (32.7 ± 89.9 μg C m^{-1} s^{-1}) and LL + GW (45.9 ± 265.1 μg C m^{-1} s^{-1}) scenarios. However, there were no statistical differences in U_{DOC} among the four scenarios considered, likely because U_{DOC} was highly variable within each scenario (coefficient of variation (CV) > 250% in all scenarios) (Figure 4A).

From the whole dataset, 40% of the cases (45 out of 112) showed $U_{DOC} > 0$ (uptake > release), while 24% of the cases (27 out of 112) showed $U_{DOC} < 0$ (uptake < release). The remaining number of the cases (36%) showed $U_{DOC} \sim 0$ (uptake ~ release). The proportion of cases with positive, negative, and nil U_{DOC} differed among scenarios ($\chi^2 = 14.2$, df = 6, $p = 0.026$). Specifically, the GW scenario showed a higher proportion of $U_{DOC} > 0$ and a lower proportion of $U_{DOC} < 0$ compared to the LL scenario (post-hoc test, $p = 0.024$) (Figure 4B). Between the other pairs of scenarios, differences in the proportion of U_{DOC} were not statistically significant.

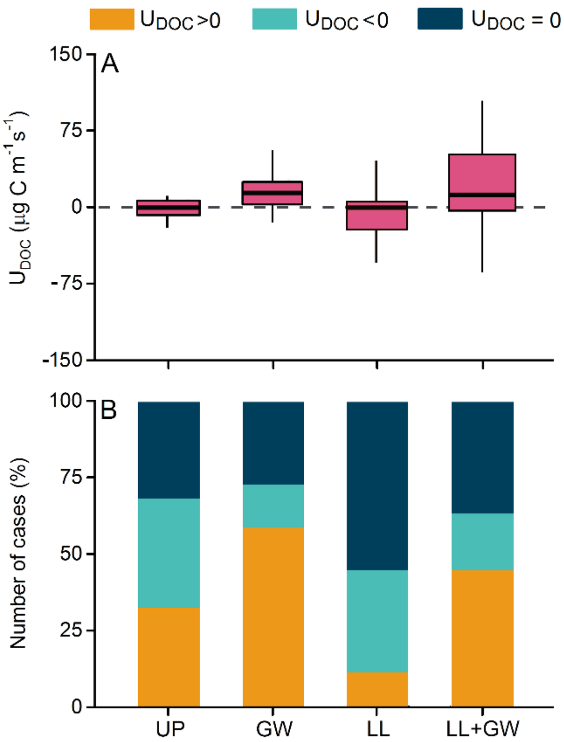

Figure 4. (**A**) In-stream net dissolved organic carbon uptake (U_{DOC}) for each scenario. Boxplots display the median, 25th, and 75th percentiles of U_{DOC} and whiskers extend to the 10th and 90th percentiles. The horizontal dashed line at $U_{DOC} = 0$ is shown as a reference. There were no differences among scenarios (Mann–Whitney test). (**B**) Relative number of cases when dissolved organic carbon (DOC) uptake prevailed over release ($U_{DOC} > 0$), DOC release prevailed over uptake ($U_{DOC} < 0$), and DOC uptake ~ DOC release ($U_{DOC} = 0$) for each scenario. Differences in the proportion of $U_{DOC} > 0$ and $U_{DOC} < 0$ were only significant between scenarios GW and LL (Tukey's test). Scenarios are: mostly upstream dissolved organic matter (DOM) inputs (UP), groundwater and upstream DOM inputs (GW), leaf litter and upstream DOM inputs (LL), and leaf litter, groundwater, and upstream DOM inputs (LL + GW).

3.3. Factors Influencing In-Stream Net DOC Uptake Variability

Variables directly related to DOM characteristics (i.e., stream DOC concentration and DOM composition) were poor predictors of U_{DOC} within scenarios because together they explained only <35% of its variability (Figure 5A). The exception was the LL + GW scenario, for which stream DOC concentration explained ~70% of U_{DOC} variability (Figure 5A). Stream DOC concentration was the only explanatory variable selected for scenarios UP and LL + GW, while the FI index was the only explanatory variable selected for the GW and LL scenarios (Table 2, Figure 5A). The BIX, HIX, and P_{DOM} metrics were not selected in any case.

The inclusion of additional environmental variables not directly related to DOM characteristics improved the explanatory power of the best-fit models for all scenarios (Figure 5B), but especially for those without leaf litter inputs. For the UP scenario, the percentage of variance explained increased from 13% to 46%, while for the GW scenario, it increased from 11% to 64% (Figure 5). For the LL and LL + GW scenarios, the goodness of fit improved <15%. The environmental variables selected within the best-fit models differed among scenarios (Table 2). Physical and hydrological variables, such as Temp, $\hat{Q}$, and Q_{GW}, were selected only for scenarios with no leaf litter inputs (UP and GW). Further, stream nitrogen concentration (either NH_4, NO_3, or DON) was selected in the best-fit models of U_{DOC} for three out of four scenarios (GW, LL, and LL + GW). The variables Q and SRP were not selected in any case.

When all data were pooled together, stream DOC concentration and H_{DOM} were the only DOM-related variables selected in the best-fit model (Table 2). Collectively, these variables explained the 26% of U_{DOC} variability, yet stream DOC concentration was the most influential one (Figure 5A). The goodness of fit of the model increased to 50% when all environmental variables were considered (Figure 5B). The only additional variable selected in the model was Q_{GW}, which explained ~25% of U_{DOC} variability (Table 2 and Figure 5B).

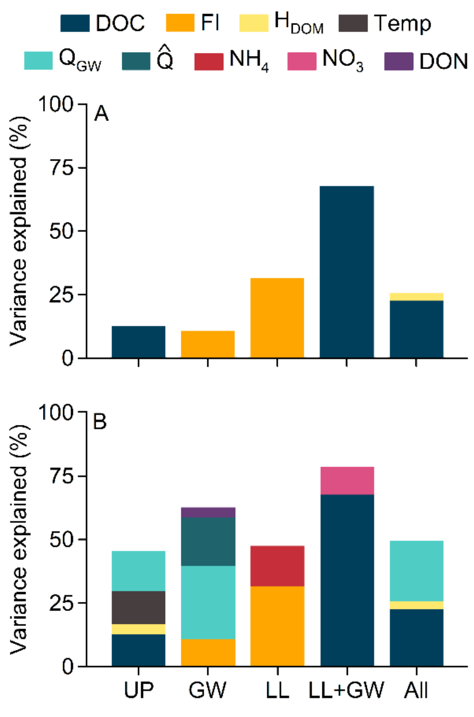

Figure 5. Variance of in-stream net dissolved organic carbon uptake (U_{DOC}) explained by each variable for the best-fit linear mixed regression model obtained for each scenario (UP, GW, LL, and LL + GW) and for all data pooled together (All). (**A**) Results from the models only including variables related to dissolved organic matter (DOM) sources, that is, stream dissolved organic carbon concentration (DOC) and stream DOM spectroscopic metrics (i.e., HIX, FI, BIX, H_{DOM}, and P_{DOM}). (**B**) Results from the models including all physicochemical variables described in Table 1. Only variables explaining >3% of the variance are shown. Abbreviations: stream dissolved organic carbon concentration (DOC); fluorescence index (FI); PARAFAC humic-like group (H_{DOM}); stream water temperature (Temp); groundwater discharge (Q_{GW}); stream discharge normalized by catchment area ($\hat{Q}$); stream concentration of ammonium (NH_4), nitrate (NO_3), and dissolved organic nitrogen (DON).

Table 2. Multiple linear regression models that better explained in-stream net dissolved organic carbon uptake (U_{DOC}) variability for each scenario and for all data pooled together. Models were performed: (1) only including variables directly related to dissolved organic matter (DOM) sources (i.e., stream dissolved organic carbon concentration and DOM spectroscopic metrics; left column) and (2) including all variables described in Table 1 (right column). All models are statistically significant ($p < 0.05$). The variance explained in each case is shown in Figure 5. Scenarios are: mostly upstream DOM inputs (UP), groundwater and upstream DOM inputs (GW), leaf litter and upstream DOM inputs (LL), and leaf litter, groundwater, and upstream DOM inputs (LL + GW).

Scenario	Model Only with DOM Variables	Model with all Variables
UP	$U_{DOC} = 28 - 71 \times DOC$	$U_{DOC} = 53 - 102 \times DOC - 126 \times H_{DOM} - 910 \times Q_{GW} + 4 \times Temp$
GW	$U_{DOC} = -788 + 328 \times FI$	$U_{DOC} = -962 + 358 \times FI + 1848 \times Q_{GW} + 5 \times \hat{Q} + 594 \times DON$
LL	$U_{DOC} = -422 + 141 \times FI$	$U_{DOC} = 480 + 270 \times FI - 54082 \times NH_4$
LL + GW	$U_{DOC} = 271 - 193 \times DOC$	$U_{DOC} = 498 - 230 \times DOC - 646 \times NO_3$
All data	$U_{DOC} = 3 - 58 \times DOC + 90 \times H_{DOM}$	$U_{DOC} = -8 - 61 \times DOC + 98 \times H_{DOM} + 2083 \times Q_{GW}$

4. Discussion

4.1. Variability of In-Stream Net DOC Uptake

Natural sources of DOM can influence in-stream DOC uptake by modulating the composition and diversity of the prevalent DOM material [7,8]. Accordingly, we found an imprinted signature in stream chemistry related to leaf litter inputs, which produced increases in humic-like DOM compounds in the stream water, and likewise in other Mediterranean streams [33,34]. Conversely, we did not detect any clear DOM signature associated with upstream or groundwater inputs. By definition, the upstream source is a mixture of DOM produced within the stream (e.g., algae and microbial exudates) and the remnants of DOM from groundwater and leaf litter inputs occurring at upstream locations and not used in the upstream reaches. Therefore, in the case of the upstream inputs, the absence of a DOM signature could be attributed to the inherent temporal variability of this source. In the case of groundwater, we attribute the lack of DOM signature to the fact that all field campaigns were conducted during relatively low flows (Table 1), which ostensibly prevent us of capturing changes in DOM composition associated with the mobilization of DOM from soils during storms [20,35,36]. Moreover, hydrological interactions at the stream–groundwater interface are complex [23,37], and hence, we cannot rule out the existence of some hydrological mixing between the stream and the groundwater, even for the net losing scenarios. Indeed, the characterization of DOM composition indicated that samples of the UP (net losing) and the GW (net gaining) scenarios contained both fresh materials from soil microbial activity and autochthonous DOM compounds [28,38], which bears the idea that there was strong groundwater–stream connectivity throughout the year in this catchment.

Despite differences in stream chemistry among scenarios, U_{DOC} was comparable and relatively low for all of them, which concurs with similar studies conducted in other headwater streams [39,40]. Nonetheless, differences in the proportion of positive, negative, and nil U_{DOC} among scenarios suggest that some DOM sources may be more prone to fuel either DOC uptake ($U_{DOC} > 0$) or release ($U_{DOC} < 0$). For instance, the predominance of $U_{DOC} > 0$ in the GW scenario indicates that DOC inputs from riparian groundwater can stimulate microbial activity and in-stream DOC uptake in headwater streams [41,42]. On the contrary, the higher proportion of $U_{DOC} < 0$ in the LL scenario highlights the importance of leaf litter as a source of available DOM to freshwater systems [35,43,44]. Taken together, these results hint the influence of different natural DOM sources on in-stream C processing, and ultimately warn the need to properly assess DOM inputs to constrain the uncertainty associated with the contribution of freshwaters on the C cycle.

Finally, in contrast to our initial expectation, we did not find evidence that a higher diversity of DOM sources influenced in-stream net DOC uptake in the stream studied. A recent review identified several potential system-specific features that could limit non-additive effects on DOM uptake in aquatic systems upon mixing of sources [18]. For example, some compounds found in very small concentrations may be protected from degradation through a dilution effect [45], while others may only be taken up in specific micro-habitats or by specific biological groups or syntrophic interactions (e.g., extra enzymatic supply by a fungal community [18]). In our case, the lack of effect of DOM source mixing on U_{DOC} may be attributed to the high variability in DOM composition observed in all scenarios, which may be limiting the detection of such effects (Figure 3B). In addition, the overall dominance of protein-like compounds in the stream DOM pool could partially buffer the interactive effect of sources with different DOM composition [18]. Interestingly, the amount of protein-like materials in the stream water was higher than those previously reported for riparian groundwater and leaf-litter leachates in the same catchment [14,46]. This finding suggests an internal microbial source of labile DOM and/or a fast DOM turnover that could homogenize the baseflow DOM pool throughout the year. Further, the fact that the U_{DOC} estimates were 10–100 times lower than gross DOC uptake reported for headwater Mediterranean streams [7] indicates fast DOC turnover rates, which supports the idea that C is quickly cycled in this system. Collectively, these observations highlight the potential of in-stream processes for changing stream DOM composition and, more broadly, reinforce the role of headwater streams as C bioreactors within catchments [6,40].

4.2. Influence of DOM Availability and Composition on In-Stream Net DOC Uptake

We found a large variability in U_{DOC} within and among scenarios. Recent works have attributed variation in DOC uptake to heterogeneities in the DOM pool and the associated microbial community [7,47]. However, results from the multiple regression models indicate that stream DOM composition barely influenced in-stream net DOC uptake in this stream (Table 2, Figure 5). An exception was observed in the LL scenario, where U_{DOC} linearly increased with increasing values of the FI index. Typically, low FI values are characteristic of terrestrial DOM sources, while high FI values are attributed to microbial DOM sources [25]. Therefore, our results seemingly indicate that, during the leaf litter period, there were shifts from plant- to microbial-derived DOM that could switch in-stream DOC cycling from net release to net uptake. These findings are in agreement with the high variability in DOM materials observed in this scenario as well as with general temporal patterns of leaf litter decomposition [47,48], thus reflecting the importance of this process to understand in-stream DOM dynamics during the leaf litter fall period.

For the scenarios without leaf litter inputs (UP and GW), the hydrological mixing between the stream and the riparian zone appears to modulate U_{DOC}. The same result was found when all scenarios were pooled together. In all the aforementioned cases, U_{DOC} was positively related to Q_{GW}, suggesting that lateral water inputs from riparian soils can promote in-stream net DOC uptake. Previous studies have documented that groundwater fluxes favor DOC assimilation in freshwaters by increasing either the resource supply [42,49] or the stream microbial meta-population [50]. Given that DOC concentration was higher in the riparian groundwater (1.40 ± 0.97 mg C L^{-1}) than in the stream water (0.80 ± 0.98 mg C L^{-1}) during all the periods studied [14], we suggest that groundwater mostly acts as a source of DOM in this stream. Moreover, the large amount of fresh microbial material observed in the stream water samples during the two scenarios with groundwater inputs (Figure 3) further indicate that this DOM can be easily used by stream biota. These results suggest that groundwater DOC supply might entail a constraint for stream metabolism and ultimately add to the growing evidence that microbial communities in Mediterranean headwater streams are C-limited [7].

Besides hydrological mixing, stream water temperature also influenced U_{DOC} variability in the UP scenario, which is consistent with the increases in heterotrophic DOC demand and microbial respiration observed during warm periods in other headwater streams [19,51,52]. Moreover, the low predictive power of some models suggest that other environmental variables not included in the analyses, such as light availability or water residence time [40,51], may also affect in-stream net DOC uptake. In our case, increases in light inputs in spring may enhance U_{DOC} by increasing the potential of DOC photodegradation [53] and/or favoring the activity of in-stream primary producers (i.e., algae), which can be a source of labile DOM to stream water [36,51]. However, it seems unlikely that variation in water residence time could account for the observed variability in U_{DOC} because mean water velocity was similar among reaches (0.32 ± 0.13 m s^{-1}). Regardless, in-stream C cycling results from the interplay of several environmental factors that affect the composition, biomass, and activity of stream microbial communities [43,51,54]. Hence, further research in this line of work is needed to understand the controls and fate of DOM in fluvial systems.

Finally, the negative relationship between U_{DOC} and both stream DOC and DIN concentrations observed in most scenarios reflects the interplay between in-stream biogeochemical processes and the amount of DOC and nutrients transported to downstream ecosystems. The interaction between in-stream C cycling and stream chemistry was especially noticeable in the LL + GW scenario, where stream DOC and NO_3 concentrations explained ~80% of U_{DOC} variability. Moreover, the LL + GW scenario also showed the highest diversity in DOM sources and the largest variability in DOM materials (Figure 3B). Together, these findings suggest that differences in the relative contribution of each DOM source among reaches could favor unbalances in DOC cycling (i.e., $U_{DOC} \neq 0$) that are mirrored in stream chemistry. For instance, fresh leaf litter might fuel fast rates of DOC leaching and N mineralization, and consequently lead to increments of stream DOC and DIN concentrations, in reaches with large amounts of litter inputs [19,35,47]. On the other hand, in reaches with less litter inputs, labile DOM from groundwater or in-stream production might reduce stream DOC and DIN concentrations by increasing DOC uptake and the associated N demand [55,56]. Accordingly, we might expect a shift from predominance of DOC release to predominance of DOC uptake along Mediterranean streams during the leaf litter period because reaches located at the forested headwaters tend to be more influenced by the riparian tree canopy than reaches located downstream [5]. While further studies are needed to confirm this hypothesis, it is ostensibly that there is a high heterogeneity in DOC processing and exports in Mediterranean streams, and that this heterogeneity is tightly coupled to changes in the dominant DOM source over time and along the fluvial network. Overall, these results reinforce the idea that a better understanding, and representation in sampling schemes, of the main DOM sources is essential to mechanistically understand C processing rates in headwater streams.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/12/6/1722/s1: Figure S1: Excitation-emission fluorescence matrices for each DOM source scenario, Figure S2: Coefficient of variance of stream DOC concentration and stream DOM spectroscopic metrics for each DOM source scenario.

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